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Reversible phosphorylation - dephosphorylation determines the localization of rab4 during the cell cycle

Peter van der Sluijs, Michael Hull, Lukas A.Huber[1], Philippe Mâle, Bruno Goud[2] and Ira Mellman

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, PO Box 3333, New Haven, CT 06510, USA, [1]European Molecular Biology Laboratory, Cell Biology Program, Meyerhofstrasse 1, W-6900 Heidelberg, Germany and [2]Department of Somatic Cell Genetics, Institut Pasteur, 25 rue de Dr Roux, 75724 Paris, France

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The ras-like GTP binding protein rab4 is the only known rab protein on endosomes that is phosphorylated during mitosis. Since a large fraction of rab4 accumulates in the cytosol in mitotic cells, we investigated the molecular mechanism controlling membrane association of rab4. We first show that human rab4 is phosphorylated by recombinant mammalian p34^{cdc2} kinase *in vitro*. Next, the actual site of phosphorylation and its functional significance were determined using stably transfected CHO cell lines producing high levels of wild type rab4 or rab4 mutants bearing alterations at Ser196, which occurs within a consensus site for p34^{cdc2} kinase phosphorylation (S^{196}PRR). Mutation of Ser196 to glutamine or aspartic acid completely prevented rab4 phosphorylation in mitotic cells and also blocked its appearance in the cytosol. Neither C-terminal isoprenylation nor carboxymethylation of rab4 was affected by the mutations or by phosphorylation. Finally, dephosphorylation and reassociation of soluble rab4 with membranes occurred upon exit of cells from mitosis. Thus, phosphorylation of Ser196 is directly responsible for the reversible translocation of rab4 into the cytosol of mitotic cells.

Key words: cell cycle/membrane attachment/p34^{cdc2}/rab4/serine phosphorylation

Introduction

Members of the *rab* family of ras-like small GTP binding proteins are the mammalian homologues of Ypt1 and Sec4, two proteins known to play critical roles in membrane transport in the secretory pathway in *Saccharomyces cerevisiae* recently reviewed in Goud and McCaffrey (1991) and Pfeffer (1992). The *rab* gene family contains at least 20 members several of which have been localized to distinct intracellular compartments. Rab6 is associated with elements of the medial Golgi and *trans*-Golgi network (Goud *et al.*, 1990), rab2 is associated with the *cis*-Golgi network (Chavrier *et al.*, 1990) and rab1 is found in both the ER and Golgi apparatus (Plutner *et al.*, 1991). The localization of different rab proteins to the various compartments of the secretory pathway suggests that each member of the family is responsible for mediating discrete transport events that either orginate or end in that compartment (Bourne, 1988).

rab proteins have also been found associated with organelles of the endocytic pathway. rab4 (rab4a) and rab5 (rab5a) are associated with early endosomes, whereas rab7 is found with late endosomes (Chavrier *et al.*, 1990; van der Sluijs *et al.*, 1991). As early and late endosomes exhibit different functions, it is perhaps not surprising that they should be associated with distinct rab proteins. Early endosomes serve as the primary site for receptor-ligand dissociation, for the transfer of discharged ligands to late endosomes and lysosomes and for the return of rapidly recycling receptors back to the plasma membrane. Recycling is thought to occur by receptor accumulation in the tubular extensions of early endosomes (Geuze *et al.*, 1987), which in some cells or after brefeldin A treatment, form a reticular network throughout the peripheral cytoplasm (Hopkins *et al.*, 1990; Hunziker *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991). In contrast, late endosomes serve as a primary site for accumulation of ligands destined for degradation in lysosomes. However, they may also play a role in receiving Golgi-derived clathrin-coated vesicles bearing newly synthesized lysosomal components (Kornfeld and Mellman, 1989).

While little is known concerning the function of the late endosome-associated rab proteins, recent *in vivo* and *in vitro* studies suggest that rab4 and rab5 control distinct, albeit incompletely understood, steps in the early endocytic pathway. rab5 appears to be involved in lateral fusion between early endosomes and possibly fusion between endosomes and incoming coated vesicles. Antibodies against rab5 block endosome fusion *in vitro* (Gorvel *et al.*, 1991) and in intact cells, expression of a rab5 mutant that does not bind GTP results in fragmentation of early endosomes and inhibits internalization and recycling during fluid phase endocytosis (Bucci *et al.*, 1992). On the other hand, overexpression of wild type, but not mutant, rab4 has no effect on initial rates of endocytosis, but does markedly decrease the accumulation of fluid phase tracers and cause a dramatic redistribution of transferrin (Tfn) receptors from early endosomes to the plasma membrane (van der Sluijs *et al.*, 1992). Accordingly, rab4 appears to play a role in the early sorting functions of endosomes.

rab4 is of further interest because it contains a consensus sequence for phosphorylation by p34^{cdc2} kinase. During metaphase, membrane traffic along the biosynthetic and endocytic pathways is significantly inhibited, resuming only after the cell has divided (Sager *et al.*, 1984; Warren *et al.*, 1984; Featherstone *et al.*, 1985). The mechanisms underlying the cessation of endocytosis at the G_2-M transition are not understood, but they are thought to involve phosphorylation and dephosphorylation, reactions perhaps initiated by p34^{cdc2} kinase (Warren, 1989). The inhibition of lateral early endosome fusion by mitotic *Xenopus* extracts and the phosphatase 1 and 2A antagonists okadaic acid and microcystin-LC, are consistent with a role for phosphorylation in regulating the endocytic pathway during the cell

cycle (Tuomikoski *et al.*, 1989; Woodman *et al.*, 1992). Moreover, these observations suggest that at least one substrate for $p34^{cdc2}$ or some other cell cycle-dependent kinase is associated with early endosomes. Conceivably, rab4 may be a target of functional importance in regulating endosome function in mitotic cells.

Consistent with this possibility are recent results showing that endogenous rab4 in HeLa cells is phosphorylated during mitosis; moreover, an increased fraction of rab4 was soluble in the cytoplasm (Bailly *et al.*, 1991). However, it remains unclear whether the phosphorylation occurs at the potential $p34^{cdc2}$ kinase site (Ser196), whether the phosphorylation is a cause or effect of the altered distribution of the protein, or whether another post-translational modification—such as a cell cycle-dependent removal of the C-terminal isoprenyl group—is actually responsible for the accumulation of rab4 in the cytosol.

To investigate the relationship between phosphorylation and membrane attachment of rab4, we have generated stable CHO cell lines overexpressing mutant or wild type rab4. This strategy enabled us to investigate under steady state conditions the mechanisms controlling rab4 association with endosomes in interphase and mitotic cells. Our results indicate that Ser196 is the unique site for phosphorylation by $p34^{cdc2}$ kinase and that phosphorylation of this site is completely responsible for the increase in soluble rab4 during mitosis.

Results

Rab4 is a substrate for recombinant human $p34^{cdc2}$ kinase in vitro

Previous results have shown that human rab4 can be phosphorylated *in vitro* by $p34^{cdc2}$ or a related kinase immunoprecipitated from HeLa cells (Bailly *et al.*, 1991). To establish that homologous mammalian $p34^{cdc2}$ is actually the kinase that phosphorylates rab4, we used an *in vitro* kinase assay with well defined components consisting of recombinant $p34^{cdc2}$, cyclin B and rab4. cDNAs encoding for the human $p34^{cdc2}$ kinase and human cyclin B was expressed using the baculovirus system in insect cells (Parker *et al.*, 1991). Infected cell extracts were then used to phosphorylate purified human rab4 protein which was produced in *Escherichia coli* (van der Sluijs *et al.*, 1991). As shown in Figure 1, rab4 was indeed a substrate for phosphorylation by $p34^{cdc2}$ kinase. No phosphorylation was observed following removal of the kinase from the Sf9 extracts by adsorption to $p13^{suc1}$ beads or by using extracts prepared from cells infected in the absence of kinase and/or cyclin B cDNAs. Although these experiments did not define the site of phosphorylation, they did demonstrate that rab4 is a substrate for mammalian $p34^{cdc2}$ kinase *in vitro*.

Cell lines stably expressing mutant and wild type rab4

To investigate the relationship between rab4 phosphorylation and membrane association during the cell cycle, it was first necessary to generate stable cell lines expressing high levels of transfected human rab4. For this purpose, we produced two cell lines overexpressing wild type rab4 or a rab4 point mutant in which the single consensus site for phosphorylation by $p34^{cdc2}$ kinase [(S/T)PXY, where X and Y are basic amino acids] (Moreno and Nurse, 1990) was

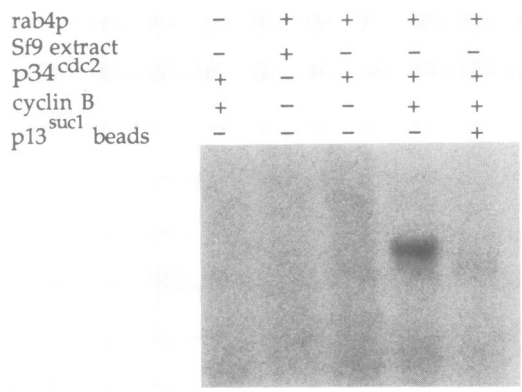

Fig. 1. Phosphorylation of rab4 by $p34^{cdc2}$ kinase *in vitro*. Purified recombinant human rab4 was incubated with Sf9 cell extracts containing recombinant human $p34^{cdc2}$ kinase and recombinant cyclin B in the presence of [γ-^{32}P]ATP. After 25 min at 30°C, rab4 was immunoprecipitated and analysed by SDS–PAGE on 12.5% gels and autoradiography. Control Sf9 cells extracts, not infected with recombinant baculovirus carrying cDNAs for $p34^{cdc2}$ kinase and recombinant cyclin B, were not able to phosphorylate rab4. Phosphorylation was observed neither in the absence of cyclin B nor when $p34^{cdc}$ kinase was depleted by preincubation with $p13^{suc1}$ beads.

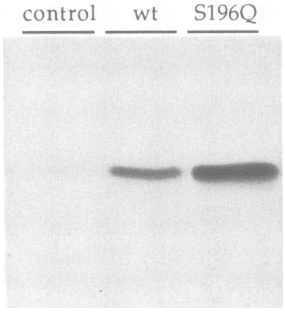

Fig. 2. Stable cell lines overexpressing wild type rab4 and rab4–S^{196}Q. Non-transfected control CHO cells and cells stably expressing wild type rab4 or the rab4–S^{196}Q mutant were washed with PBS. The cells were homogenized in 10 mM triethanolamine pH 7.4, 1 mM EDTA, 1 mM HOAc, containing 250 mM sucrose. The homogenate was centrifuged at 600 *g* for 15 min to generate a postnuclear supernatant. Samples were normalized for protein content and analysed by SDS–PAGE and Western blotting using a rabbit antiserum against recombinant rab4.

removed by changing the serine at position 196 (SPRR) to glutamine (rab4–S^{196}Q). Chinese hamster ovary (CHO) cells were then transfected with the expression vector pFRSV, which contains a mutant dehydrofolate reductase gene that allows for amplification of expression levels in increasing concentrations of methotrexate (MTX) (Miettinen *et al.*, 1989).

Transfectants were selected in 60 μM MTX and found to exhibit >80-fold more mutant and wild type human rab4 relative to the endogenous hamster rab4 (Figure 2). The overall morphology and growth rate of transfectants were comparable to non-transfected control cells. Overexpressed wild type rab4 and rab4–S^{196}Q were also correctly targeted to transferrin receptor-containing early endosomes, as analysed by electron microscopy and confocal immunofluorescence microscopy (van der Sluijs *et al.*, 1992) (see below). Moreover, most of the transfected rab4–S^{196}Q mutant and wild type rab4 was membrane-associated, with 90% of wild type rab4 and 94% of rab4–S^{196}Q found by

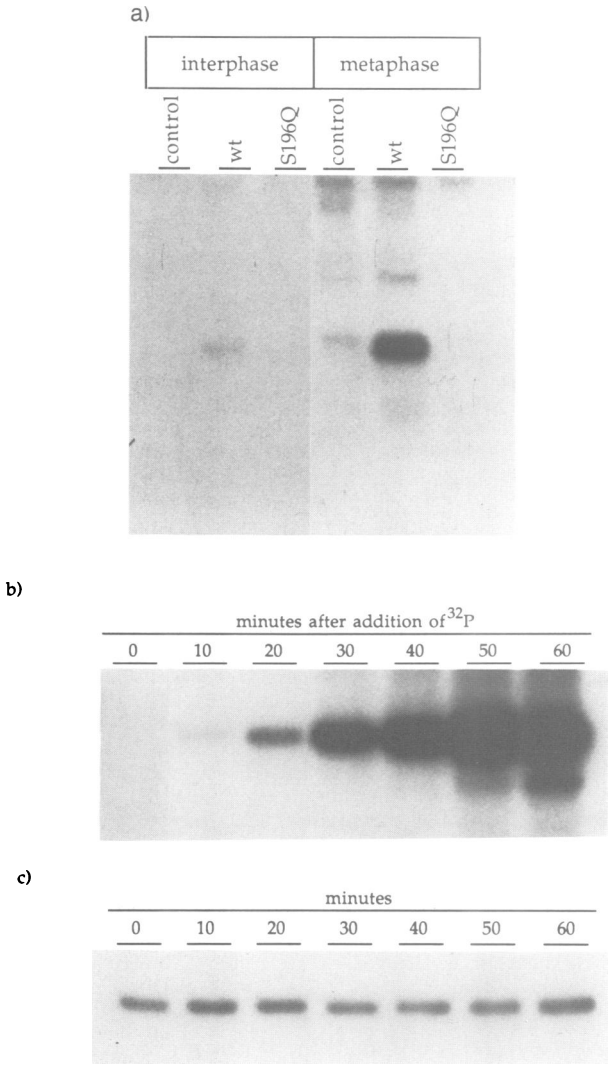

Fig. 3. Rab4 is phosphorylated on Ser196 in mitotic cells. Control CHO cells or cells overexpressing wild type rab4 or the rab4−S^{196}Q mutant were synchronized by thymidine treatment and blocked in prometaphase with 40 ng/ml nocodazole. Mitotic cells were collected by mechanical shake off and starved for 30 min in labelling medium. (**A**) The cells were labelled for 30 min with $^{32}P_i$ and lysed with 0.5% Triton X-100. rab4 was immunoprecipitated with a polyclonal antiserum and analysed by SDS−PAGE and autoradiography. (**B**) Mitotic cells were starved and labelled for various periods of time with $^{32}P_i$ and lysed with detergent. rab4 was immunoprecipitated and analysed by SDS−PAGE and autoradiography. (**C**) Same experiment as (**B**), but the cells were not labelled with $^{32}P_i$ prior to detergent lysis and immunoprecipitation. After SDS−PAGE, proteins were transferred to nitrocellulose and analysed by Western blotting using the affinity purified anti-peptide antibody.

Western blot to pellet following centrifugation at 145 000 *g* (see below). Together these results suggested that the relatively high levels of expression did not saturate the capacity of early endosomes to bind rab4.

Total cellular rab4 is phosphorylated on Ser196 during mitosis

Previous studies using HeLa cells demonstrated that during mitosis endogenous rab4 was phosphorylated and became increasingly cytosolic (Bailly *et al.*, 1991). This result was potentially relevant to the cessation of endocytosis in mitotic cells, since soluble rab4 would not be expected to mediate any endosome-associated activities. However, it remained unclear whether the phosphorylation occurred at the potential $p34^{cdc2}$ kinase site (Ser196), whether phosphorylation was even necessary for the change in membrane association and to what extent the entire cellular pool of rab4 was affected. To investigate these issues, the distribution and phosphorylation of wild type rab4 and the rab4−S^{196}Q mutant were determined as a function of cell cycle using the stably transfected CHO cells.

Cells were synchronized using a thymidine block followed by arrest in prometaphase by incubation in nocodazole (Suprynowicz and Gerace, 1986). Prometaphase- or interphase-enriched populations were then labelled for 30 min with $^{32}P_i$, lysed in detergent and rab4 was immunoprecipitated from cells expressing comparable amounts of wild type rab4, rab4−S^{196}Q or from non-transfected controls. No phosphorylated rab4 was detected in interphase cells in the non-transfected controls or in cells expressing the rab4−S^{196}Q mutant (Figure 3a). While a small amount of phosphorylated rab4 was observed in interphase cells overexpressing wild type rab4, the amount of phosphorylated wild type rab4 in mitotic cells increased dramatically (Figure 3b). In contrast, almost no phosphorylation was detected in cells expressing rab4−S^{196}Q mutant; the small amount of labelled rab4 detected was not different from that found in non-transfected control cells and presumably reflected labelling of the endogenous wild type CHO rab4 (Figure 3b). Together, these results showed that rab4 is uniquely phosphorylated in mitotic cells on Ser196, the predicted $p34^{cdc2}$ kinase phosphorylation site.

We next established the kinetics of wild type rab4 phosphorylation by labelling mitotic cells for increasing lengths of time with $^{32}P_i$. Phosphorylation was rapid, being detectable within 10 min after adding the $^{32}P_i$ and reaching a maximum after 50 min (Figure 3b). Since the total amount of wild type rab4 was found by Western blot to be constant over this time-course (Figure 3c), the increase in $^{32}P_i$ labelling must have resulted from an increased amount of phosphorylation. Pulse−chase experiments indicated further that the rate of turnover of rab4 was identical in transfected interphase and mitotic cells (not shown).

To investigate which fraction of the total cellular rab4 pool became phosphorylated during mitosis, we analysed the electrophoretic mobility of rab4 on high resolution 2D gels. Wild type rab4 transfectants were synchronized in prometaphase or in interphase and lysed in detergent. Lysates were resolved by 2D gel electrophoresis and individual transfers probed with [α-^{32}P]GTP to visualize all GTP binding proteins and with the anti-peptide antiserum to detect rab4. In interphase extracts, the rab4-containing region of the gel contained several proteins that bound labelled GTP (Figure 4a), but only one of these was reactive with the anti-rab4 antibody (Figure 4b, arrowhead). In mitotic extracts, the position of rab4 was quantitatively shifted to a more acidic position as detected by GTP binding (Figure 4c; small versus large arrowheads) and Western blotting (Figure 4d). The shift was detected by aligning the invariant GTP-binding proteins. Since other post-translational modifications of rab4 were not altered during the cell cycle (see below), this observation strongly suggested that, in mitotic cells, virtually the entire cellular pool of rab4 was phosphorylated and thus exhibits a slightly more acidic isoelectric point.

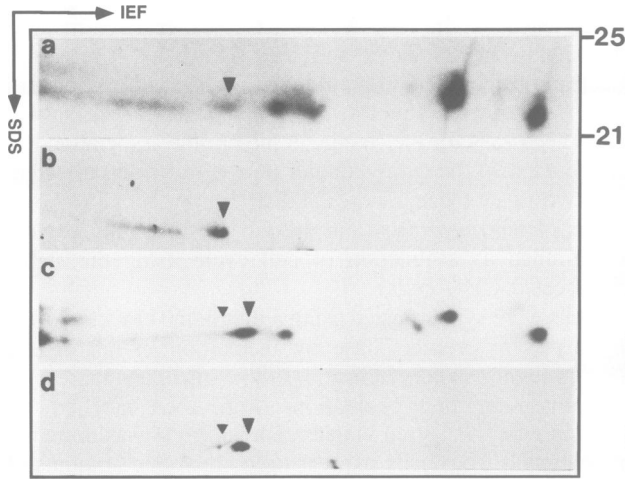

Fig. 4. The entire pool of rab4 exhibits a more acidic isoelectric pH in mitotic cells. Wild type rab4 overexpressing cells were synchronized in interphase (**a** and **b**) or prometaphase (**c** and **d**) lysed in detergent and subjected to high resolution 2D gel electrophoresis. Proteins were transferred to nitrocellulose and the filters probed with [α-^{32}P]GTP (a and c), stripped and then probed by Western blot with affinity purified anti-rab4 peptide antibody (b and d).

Phosphorylation is necessary for accumulation of cytosolic rab4 during mitosis

To determine whether phosphorylation of Ser196 was involved in controlling the distribution of rab4 during the cell cycle, we next analysed the localization of wild type rab4 and rab4−S^{196}Q in mitotic and interphase cells. Transfected CHO cells were synchronized and interphase or mitotic populations labelled with $^{32}P_i$. The cells were homogenized and post-nuclear supernatants were subsequently fractionated into soluble (S) and total membrane pellet (P) fractions by high speed centrifugation. Rab4 was immunoprecipitated from both fractions and analysed by SDS−PAGE and autoradiography. In interphase cells, the relatively small amounts of ^{32}P-labelled rab4 in cells overexpressing human wild type rab4 were found predominantly (>90%) in the soluble fraction (Figure 5A). Presumably this small amount of labelled rab4 was due to incomplete removal of mitotic cells from the interphase cell populations. In contrast, in mitotic cells overexpressing wild type rab4, there was a dramatic increase in the amount of ^{32}P-labelled rab4 again accompanied by a significant increase in the fraction of the labelled protein found in the cytosol (68%). As expected, the small amount of endogenous ^{32}P-labelled rab4 was found in the soluble pool in non-transfected controls and in the rab4−S^{196}Q transfectants.

The distribution of total rab4 protein was next determined by quantitative Western blotting of both the soluble and pellet fractions. As shown in Figure 5B, the large majority (90−95%) of endogenous rab4, transfected human wild type rab4 and the rab4−S^{196}Q mutant was found membrane-associated in interphase cells. A second, lower molecular weight band was sometimes observed in the membrane fractions, reflecting a presumptive proteolytic product of rab4 (not shown). More importantly, while >75% of the transfected or endogenous wild type rab4 was found in the cytosol, the rab4−S^{196}Q mutant remained 95% membrane-bound. Thus, the phosphorylation of Ser196 by p34^{cdc2} or a related kinase was required for the accumulation of rab4 in the cytosol of mitotic cells.

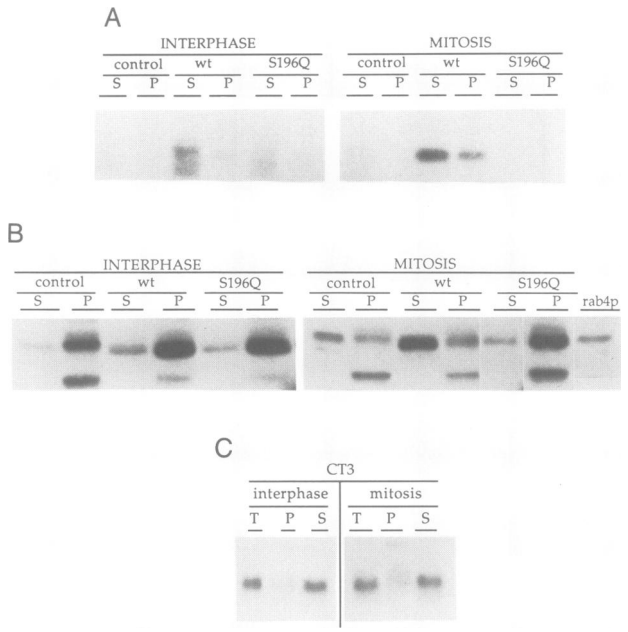

Fig. 5. Phosphorylation of rab4 results in its accumulation in the cytosol. Control cells, wild type rab4, rab4−CT3, rab4−S^{196}Q and rab4−S^{196}D cells were synchronized and labelled with $^{32}P_i$. The cells were homogenized and fractionated by high speed centrifugation. rab4 was immunoprecipitated from membrane pellet (P) and high speed supernatant (S) fractions (**A**) or from detergent lysates (**D**) and then analysed by SDS−PAGE. Alternatively, unlabelled cells were homogenized and fractionated into membrane pellet and high speed supernatant fractions for Western blotting using anti-rab4 peptide antibody (**B** and **C**). To better visualize endogenous CHO rab4, four times the amount of sample was loaded in the control lanes. On occasion, the anti-peptide antibody also recognized a band of ~18 kDa that was not detected with the antibody generated against the recombinant protein.

Although phosphorylation of rab4 may trigger its dissociation from endosomes, we have been unable to observe rab4 phosphorylation using partially purified membranes incubated with recombinant p34^{cdc2} kinase *in vitro*. Because small GTP binding proteins are thought to cycle between membrane-bound and soluble states during the course of their activities (Bourne *et al.*, 1991), we investigated whether phosphorylation could occur on the cytoplasmic pool of rab4 and possibly act by preventing its rebinding. To determine whether soluble rab4 could be phosphorylated in mitotic cells, we generated a CHO cell line expressing a rab4 mutant bearing a deletion of its consensus site (C-G-C) for C-terminal isoprenylation (rab4−CT3). As shown in Figure 5C, >90% of the rab4−CT3 mutant remained in the soluble fraction in both interphase and mitotic cells. Nevertheless, rab4−CT3 was phosphorylated in mitotic cells to the same extent as wild type rab4 (Figure 5D). Thus, membrane association is not necessary for the phosphorylation of rab4, suggesting that this modification may act to prevent re-association of rab4 during the soluble phase of its cycle.

We next determined whether the simple introduction of a negatively charged residue at position 196 was sufficient to prevent membrane association of rab4 in interphase or mitotic cells. For this purpose, we generated a rab4 mutant in which Ser196 was replaced by aspartic acid, which sometimes acts as a structural analogue of phosphoserine (Casanova *et al.*, 1990; Jans *et al.*, 1991). We used confocal

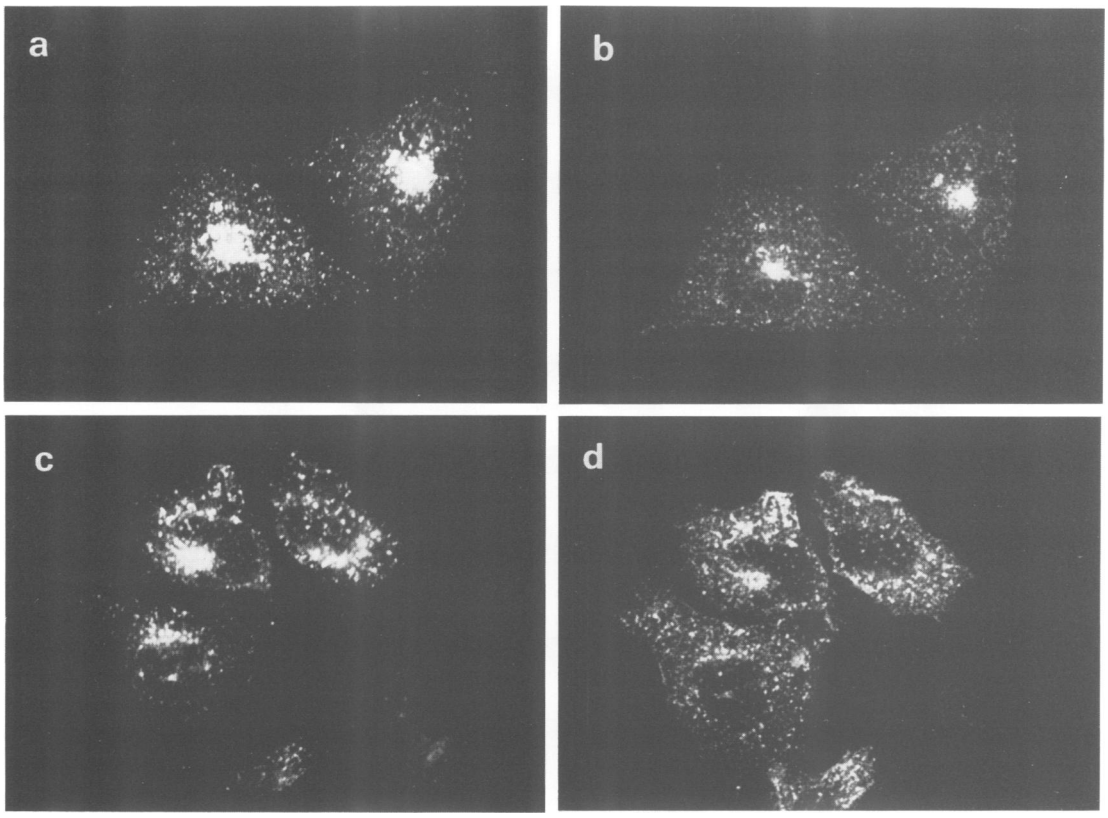

Fig. 6. Localization of rab4 wild type and the rab4−S^{196}D mutant in interphase cells. Double transfectants expressing rab4−S^{196}D and the human transferrin receptor (**a** and **b**) or wild type rab4 and the human transferrin receptor (**c** and **d**) were plated on glass coverslips and fixed with paraformaldehyde. The cells were permeabilized and incubated with affinity-purified rabbit anti-rab4 antiserum, the anti-human transferrin receptor monoclonal antibody OKT9 and stained with TRITC-conjugated donkey anti-rabbit IgG (a and c; rab4) or FITC-conjugated goat anti-mouse antibody (b and d; Tfn-R). The cells were analysed by confocal microscopy.

immunofluorescence microscopy to investigate the localization of the mutant rab4−S^{196}D in CHO cells that also stably expressed the human transferrin receptor. rab4−S^{196}D (Figure 6a) and wild type rab4 (Figure 6c) largely, but not completely, co-localized with the transferrin receptor (Figure 6b and d). This colocalization was more clearly illustrated by alignment of fluorescence patterns and consequent colour shift after merging the confocal images (not shown). We also used subcellular fractionation to analyse the distribution of the rab4−S^{196}D mutant in interphase and during mitosis. As demonstrated for rab4−S^{196}Q (Figure 5B) >95% of the rab4−S^{196}D mutant remained membrane-bound in both interphase and mitotic cells (not shown). As expected, rab4−S^{196}D was not phosphorylated during mitosis (Figure 5D). Thus, the presence of a negative charge at position 196 did not by itself result in the accumulation of soluble rab4.

C-terminal modifications of rab4 remain unchanged during the cell cycle

Having established that phosphorylation of Ser196 was necessary for generating the soluble pool of rab4 in mitotic cells, we next determined whether any other post-translational modifications that affect membrane association were regulated during the cell cycle. It is well known that all ras-like GTP-binding proteins have one or more C-terminal cysteine residues that are isoprenylated often with farnesyl or geranylgeranyl moieties, recently reviewed in Gibbs (1991). Similarly, one of the cysteines in the C-G-C motif of rab4 may be further modified by carboxymethylation (Farnsworth *et al.*, 1991). A cell cycle-dependent alteration in either or both of these modifications, perhaps secondary to the phosphorylation of Ser196, may actually be responsible for alterations in membrane attachment.

To address whether the C-terminal isoprenylation of rab4 is regulated during the cell cycle, we labelled mitotic or interphase cells with [^{3}H]mevalonolactone in the presence of lovastatin. This drug is a competitive inhibitor of HMG-CoA reductase and thus decreases intracellular stores of mevalonic acid, the precursor for isoprenyl groups. After labelling, the cells were homogenized and the post-nuclear supernatant was fractionated in membrane and cytoplasmic fractions from which the rab4−S^{196}Q mutant and wild type rab4 were immunoprecipitated and analysed by SDS−PAGE and fluorography.

In interphase cells, both wild type and mutant rab4 were isoprenylated (Figure 7a, left panel). Although a relatively large fraction of both proteins was found in the soluble pool, this was likely to have resulted from overnight labelling in the presence of lovastatin, as has been previously observed for rab proteins (Johnston *et al.*, 1991; Kinsella and Maltese, 1992) and ras (Hancock *et al.*, 1989).

In mitotic cells, wild type rab4 and rab4−S^{196}Q were also isoprenylated to similar extents (Figure 7a, right panel). In this instance, however, soluble protein was only found in the case of wild type rab4, which was also subject to phosphorylation on Ser196. 82% of the ^{3}H-labelled wild type rab4 was found in the cytosol while 78% of the ^{3}H-

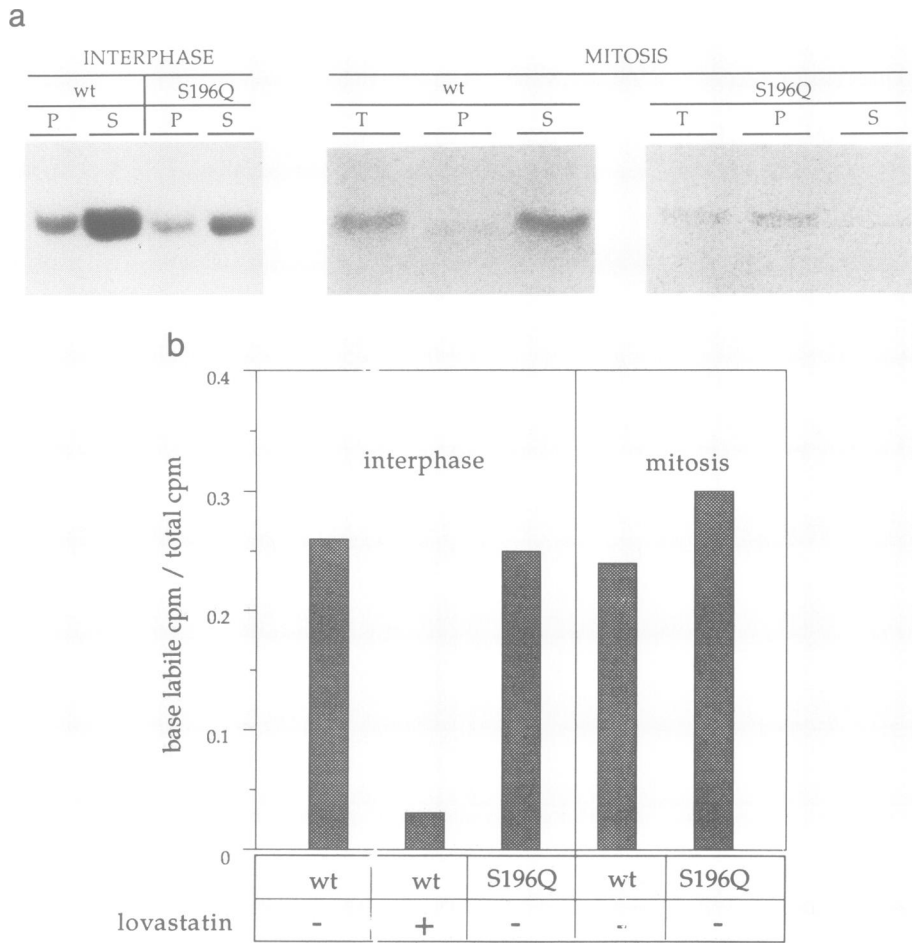

Fig. 7. Wild type rab4 and rab4−S^{196}Q are isoprenylated and carboxymethylated during mitosis. (**a**) To measure isoprenylation, wild type rab4 and rab4−S^{196}Q cells were labelled in interphase and mitosis with RS-[5-^{3}H(N)]mevalonolactone. Cells were homogenized, to give a total (T) post-nuclear supernatant, from which membrane pellets (P) and soluble cytosol (C) fractions were generated by high speed centrifugation. rab4 was immunoprecipitated from both fractions and analysed by SDS−PAGE and fluorography. Exposure of the interphase bands was 5 days and for the mitotic bands, films were exposed for 35 days. (**b**) Carboxymethylation of wild type rab4 and rab4−S^{196}Q cells was measured in interphase (in the absence or presence of 100 μM lovastatin) and mitosis with L-[methyl-^{3}H]methionine. rab4 was immunoprecipitated from lysates and visualized after SDS−PAGE and fluorography. The bands corresponding to labelled rab4 were excised from the dried gel and the amount of radioactivity due to carboxymethylation was assessed by determining the fraction of alkali-labile ^{3}H. See Materials and methods for details.

labelled rab4−S^{196}Q remained membrane-bound. A greater fraction of [^{3}H]rab4−S^{196}Q was membrane-associated in mitotic rather than in interphase cells because the mitotic cells were incubated in lovastatin and [^{3}H]mevalonolactone for only 3 h; 18 h incubations were used for interphase cells. After correcting for the shorter labelling time, we found that the same level of isoprenylation occurred on wild type rab4 and the rab4−S^{196}Q mutant in interphase and mitotic cells.

Carboxymethylation of ras-like GTP binding proteins with a C-terminal CAAX motif is also essential for efficient binding to membranes (Hancock *et al.*, 1991). Although small GTP binding proteins such as rab4, which terminate with a CXC sequence are carboxymethylated, it is not known whether this modification is required for membrane association or if it is regulated during the cell cycle. We addressed these questions by labelling interphase and mitotic cells overexpressing wild type rab4 or rab4−S^{196}Q with [^{3}H]methylmethionine, a precursor that results in transfer of an alkaline-labile methyl group to free carboxy residues, but also in incorporation into methionine residues of the nascent chain. After labelling, the cells were lysed, rab protein immunoprecipitated and analysed by SDS−PAGE and fluorography. The fluorograph was used as a template to cut out the radioactive bands and carboxymethylation was determined by alkaline hydrolysis.

As shown in Figure 7b, carboxymethylation occurred to the same extent in interphase and mitotic cells for both wild type rab4 and rab4−S^{196}Q with ratios of alkaline labile:total c.p.m. ranging between 0.25 and 0.35. Since rab4 contains three methionine residues, a ratio of 0.25 would be predicted if one [^{3}H]methyl group was incorporated per molecule of wild type or mutant rab4. It was very probable that the carboxymethylation occurred at the C-terminal C-G-C motif and not on internal acidic amino acids, since cells incubated in lovastatin prior to labelling with [^{3}H]methyl-methionine failed to incorporate any alkaline-labile radio-activity (Figure 7b). Thus, as previously shown for lamin B, isoprenylation is likely to be a prerequisite for carboxy-methylation (Kitten and Nigg, 1991).

Taken together, these results indicate that neither isoprenylation nor carboxymethylation of rab4 was altered as a function of cell cycle. Accordingly, it is apparent that

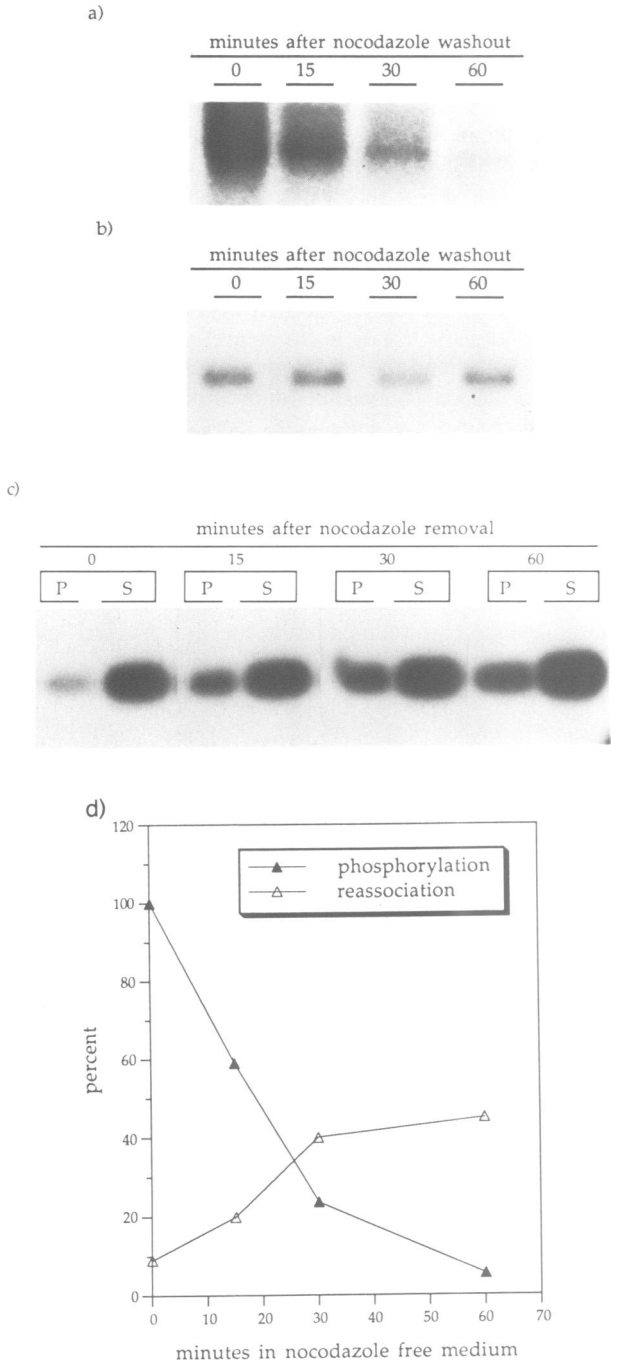

Fig. 8. Phosphorylation and membrane association of rab4 are reversible. Mitotic cells were collected after mechanical shake off, starved for 30 min and half of the cells reincubated for 30 min in $^{32}P_i$-containing labelling medium. Cells were washed and then incubated in $^{32}P_i$-containing medium in the absence of nocodazole to permit their exit from mitosis. A second aliquot of the cells was subjected to the same protocol, except that cold phosphate was substituted for $^{32}P_i$. Samples were taken 0–60 min after nocodazole removal and lysed in detergent. rab4 was immunoprecipitated from both sets of lysates and electrophoresed in 12.5% gels. (**a**) $^{32}P_i$-labelled rab4 was analysed by autoradiography of the dried gel. (**b**) Total rab4 was determined from the unlabelled cell lysates by Western blotting. (**c**) The subcellular distribution of rab4 after nocodazole removal was monitored by Western blotting of membrane and cytoplasmic fractions that were prepared from post-nuclear supernatants. (**d**) Intensities of radioactive bands in panels a and c were quantified by densitometry and plotted as a function of time in nocodazole-free medium.

phosphorylation of Ser196 is a necessary prerequisite for the accumulation of soluble rab4 in mitotic cells.

Phosphorylation and membrane association of rab4 are reversible

If phosphorylation of Ser196 and the accumulation of soluble rab4 in mitotic cells reflects a cell cycle-dependent regulation of rab4 function, one would expect both events to be reversible as cells leave metaphase, enter anaphase and reduce their levels of active $p34^{cdc2}$ kinase and cyclin (Murray *et al.*, 1989). To determine whether this was indeed the case, synchronized CHO cells expressing wild type rab4 were arrested in prometaphase using nocodazole, labelled for 30 min with $^{32}P_i$ (Figure 3b), washed at 0°C and then cultured at 37°C in nocodazole-free medium with $^{32}P_i$. Samples were taken after various periods of time, mitotic indexes determined by DNA staining with Hoechst 33258 and rab4 was immunoprecipitated. As shown in Figure 8, the amount of ^{32}P-labelled rab4 decreased rapidly, with only 5% remaining after 60 min (panel a). Since the total amount of rab4 protein remained constant over this time interval (Figure 8b), it was probable that the loss of radiolabel reflected the hydrolysis of the phosphate on Ser196. The mitotic index of the cells decreased in parallel with the amount of [^{32}P]rab4 recovered.

Finally, we examined whether the dephosphorylation of rab4 correlated with its reassociation with endosomes. Prometaphase-arrested cells were washed and then cultured in nocodazole-free medium for 0–60 min, homogenized and centrifuged to obtain soluble and membrane pellet fractions. The distribution of rab4 between the two fractions was then determined by Western blotting using the rab4 anti-peptide antibody. As shown in Figure 8c, the amount of membrane-bound rab4 increased by 4-fold. However, membrane reassociation was both slower and possibly less efficient than dephosphorylation (Figure 8d). Even after 60 min, a large fraction (62%) of dephosphorylated rab4 remained in the soluble pool. Thus, it seems apparent that although both phosphorylation and membrane association are reversible, the dephosphorylation of Ser196 was not entirely sufficient to permit the re-binding of soluble rab4 to endosomes. Conceivably, membrane attachment requires one or more additional rate limiting events following dephosphorylation.

Discussion

Small GTP binding proteins of the rab family are thought to play essential roles in the control of vesicular transport in animal cells. Although their precise activities remain to be defined, overexpression of rab4 or rab5 in fibroblasts showed that these two early endosome-associated rab proteins control distinct events on the endocytic pathway (Bucci *et al.*, 1992; van der Sluijs *et al.*, 1992). Moreover, the fact that rab4 is phosphorylated during mitosis (Bailly *et al.*, 1991) makes this protein of additional interest as a candidate mechanism for the regulation of endocytosis in mitotic cells. In this paper, we have begun to address this issue by investigating the relationship between rab4 phosphorylation and membrane attachment during different stages of the cell cycle. Our results strongly suggest that all cellular rab4 is phosphorylated on its single consensus site by $p34^{cdc2}$ kinase and that phosphorylation is a necessary prerequisite for the accumulation of soluble rab4 in mitotic cells.

Control of endosome attachment of rab4 during the cell cycle

Our strategy was to stably overexpress rab4 in CHO cells because the level of endogenous rab4 is too low to be conveniently monitored and because CHO cells are easy to synchronize. In addition, this approach allowed us to directly test the role of phosphorylation in membrane attachment using cell lines expressing mutant rab protein. In fact, both mutants in which the presumptive $p34^{cdc2}$ kinase phosphorylation site (Ser196) was changed to glutamine or aspartic acid failed to be phosphorylated in mitotic cells. Moreover, these two mutants remained >90% membrane-bound throughout the cell cycle. This is in direct contrast to transfected (and endogenous) wild type rab4, which was both heavily phosphorylated in metaphase-arrested cells and found almost quantitatively in the cytosol.

The fact that both membrane-bound and soluble rab4 retained their C-terminal isoprenyl and carboxymethyl modifications strongly suggested that the phosphorylation of Ser196 was uniquely responsible for regulating membrane association during the cell cycle. Nevertheless, as is true for other ras-like GTP-binding proteins, stable membrane attachment of rab4 was dependent on the presence of either or both of these modifications (Hancock *et al.*, 1991). Removing the last three amino acids from rab4 (rab4−CT3), which contain the probable site (C-G-C) for addition of a geranylgeranyl group (Johnston *et al.*, 1991) resulted in a mutant protein that remained soluble even in interphase cells. Consequently, we cannot eliminate the possibility that dissociation of membrane-bound rab4 in mitosis may occur as a consequence of hydrolysis of a geranylgeranyl group. However, its readdition would have to occur immediately following the translocation of rab4 to the cytosol. In this event, phosphorylation would serve to prevent the membrane binding of soluble but isoprenylated rab4. This mechanism appears unlikely, however, since there is no evidence for rapid turnover of isoprenyl groups on rab4 or other ras-like GTP binding proteins (Hancock *et al.*, 1989).

While isoprenylation may be required for stable membrane attachment, the fact that phosphorylation of Ser196 exerts a dramatic effect on rab4 localization, demonstrates that isoprenylation is not the only determinant. One or more interactions that are disrupted by phosphorylation of Ser196 must also be involved in the initial targeting or continued binding of rab4 to endosomes. Interestingly, Ser196 is 13 residues away from the rab4 C-terminus and thus falls within its 'hypervariable domain' (Chavrier *et al.*, 1991). In ras, as well as in at least two other rab proteins, this domain contains sequence information that is required for targeting to appropriate intracellular destinations (Hancock *et al.*, 1990; Chavrier *et al.*, 1991). Despite the fact that phosphorylation regulates the cell cycle-dependent localization of rab4, it is clear that Ser196 itself is not absolutely required for targeting to early endosomes. Even when changed to glutamine or aspartic acid, rab4 continues to be targeted to endosomes, as visualized by both confocal microscopy and immunoelectron microscopy (van der Sluijs *et al.*, 1992). Moreover, these results suggest that the critical interaction disrupted by phosphorylation of Ser196 was probably specific to a phosphoserine and not simply to an increase in net negative charge at this residue.

It is also interesting to note that up to 80-fold overexpression of either wild type or mutant rab4 had no appreciable effect on either the fraction of membrane-bound rab4 or on its targeting to Tfn receptor-containing early endosomes. This is in contrast to results obtained for rab5 in which overexpression led to increased accumulation of soluble protein (Gorvel *et al.*, 1991). Conceivably, this difference was due to differences between rab proteins or due to the fact that the rab5 experiments utilized a transient, vaccinia virus-based expression system. In any event, the fact that overexpressed rab4 remained largely membrane-bound suggests that there may be an excess of putative rab4 receptors on endosomes. Alternatively, the rab4 receptor may be inherently 'catalytic' in nature, with rab4 binding only transiently and the receptor mediating the anchoring of rab4 in the endosomal membrane, perhaps via rab4's isoprenyl group.

Two mechanisms could explain the accumulation of soluble rab4 in mitotic cells: phosphorylation may cause the dissociation of membrane-bound rab4 from endosomes or prevent the membrane binding of rab4 from a pre-existing soluble pool. While we have been unable to distinguish between these possibilities, we favour a mechanism whereby phosphorylation blocks membrane attachment. First, using partially purified endosome fractions isolated from interphase cells, we have thus far been unable to either phosphorylate or dissociate wild type rab4 by incubation with recombinant $p34^{cdc2}$ kinase and cyclin B (P.van der Sluijs and I.Mellman, unpublished observations). The membranes did not inhibit phosphorylation since $p34^{cdc2}$ kinase was perfectly capable of phosphorylating recombinant rab4 in the presence of the membrane fraction. Secondly, since the soluble rab4−CT3 mutant was phosphorylated in mitotic cells, it is apparent that activated $p34^{cdc2}$ or a related kinase can recognize the cytosolic form of protein. Finally, by analogy to the Sec4 protein in yeast, mammalian rab proteins are likely to exhibit repetitive cycles of membrane association and dissociation concomitant with their function (Bourne, 1988). Consequently, targeting of rab4 to endosomes might be disrupted if, in mitotic cells, a cdc2 kinase were to act while rab4 was in the cytosolic phase of its predicted activity cycle. Whether membrane binding is blocked due to the failure of phosphorylated rab4 to be recognized by an endosome-associated rab4 receptor or due to an alteration in its interaction with a cytosolic complex, which either prevents or is required for membrane binding, is unknown. The lag observed as cells exit metaphase between the rapid dephosphorylation of rab4 versus its slower reattachment to membranes is consistent with the involvement of an additional regulatory step, such as the assembly or disassembly of a rab4-containing complex. Conceivably, such a complex might include proteins such as GDP dissociation inhibitor (GDI) as recently shown for soluble rab3a and Sec4 (Sasaki *et al.*, 1991).

Phosphorylation and the function of small GTP binding proteins

In addition to rab4, several small GTP binding proteins are phosphorylated, presumably in their C-terminal domains. The ER−Golgi-associated protein rab1 is the only other known rab that is phosphorylated in mitotic cells, although phosphorylation does not elicit as dramatic a change in intracellular localization as we have found for rab4 (Bailly *et al.*, 1991). rap1a and rap1b are substrates for protein kinase A and are phosphorylated on a serine residue

immediately adjacent to the C-terminal cysteine that is involved in membrane association (Lapetina *et al.*, 1989; Quilliam *et al.*, 1991). Interestingly, phosphorylation of rap1A inhibits its binding to cytochrome b_{558}, a component of the plasma membrane NADPH oxidase system (Bokoch *et al.*, 1991). Phosphorylation of rap1B increases the soluble fraction of this protein (Lapetina *et al.*, 1989) and in platelets controls its association with the cytoskeleton (Fischer *et al.*, 1990). rap1B phosphorylation also stimulates the action of its guanine nucleotide dissociation stimulator (GDS) protein, stabilizing rap1B in the GTP-bound state (Hata *et al.*, 1991). Thus, phosphorylation of both rap1a and rap1b alters their interactions with other proteins.

While phosphorylation is not known to alter any rab4-associated function except membrane attachment, it seems probable that dissociation of the protein from endosomes would be sufficient to prevent it from conducting any endosome-specific functions. However, it is possible that phosphorylation also more directly inhibits a rab4-associated activity since Ser196 appears to be a residue that is important for the biological activity of the protein. We have recently found that overexpression of wild type rab4 in CHO cells results in a marked decreased in the accumulation of markers of fluid phase endocytosis and also significantly disrupts the pathway of Tfn receptor recycling (van der Sluijs *et al.*, 1992). In control cells, >75% of cellular Tfn receptors are found in endosomes; after rab4 expression, however, these receptors are found predominantly on the plasma membrane. Interestingly, substitution of Ser196 with either glutamine or aspartic acid prevents both of these phenotypic alterations, suggesting that this region of rab4 plays an important role in mediating its activities, perhaps by mediating its interactions with other proteins. Accordingly, it has been impossible to use these mutants to determine whether elimination of the $p34^{cdc2}$ kinase site from rab4 had any effect on the cessation of endocytosis in mitotic cells.

Phosphorylation and control of membrane traffic during the cell cycle

Over the past several years, it has become clear that phosphorylation-controlled interactions constitute a general theme responsible for regulating membrane-associated events during the cell cycle (recently reviewed in Moreno and Nurse, 1990; Freeman *et al.*, 1992). One of the best studied example is the phosphorylation of lamin B, by $p34^{cdc2}$ or a related kinase, an event that is a prerequisite for disassembly of the nuclear lamina during mitosis in higher eukaryotes (Heald and McKeon, 1990; Peter *et al.*, 1991). Unlike rab4 or ras, lamin B is also demethylated during mitosis (Chelsky *et al.*, 1987). Given the well known inhibition of membrane traffic in mitotic cells (Warren, 1985), it seems probable that phosphorylation of a variety of other critical proteins may play similar roles in regulating transport on the endocytic and secretory pathways. Indeed, indirect or direct phosphorylation of endosomes by a $p34^{cdc2}$ kinase is capable of blocking lateral fusion of endosomes *in vitro* (Tuomikoski *et al.*, 1989). Aside from rab4, however, no endosomal substrates for phosphorylation in mitotic cells have yet been identified.

It has now become important to determine the other cytosolic and/or membrane components whose interactions with rab4 are regulated by cell cycle-dependent phosphorylation. Not only are these proteins likely to be of importance with respect to rab4 function, but their identification will also help determine the significance of such phosphorylation events in controlling intracellular membrane traffic.

Materials and methods

Cell culture and synchronization

CHO cells were grown in αMEM, supplemented with 10% fetal calf serum, 100 U/ml ampicillin and 100 μg/ml streptomycin. Transfected cells were maintained in medium containing 60 μM methotrexate. Mitotic cells were obtained after a modification of published procedures (Suprynowicz *et al.*, 1986). A confluent 10 cm dish was trypsinized and its contents were seeded in a 24.5 × 24.5 cm plate (Nunclon). After 48–55 h, thymidine (Sigma Co., St Louis, MO) was added to a final concentration of 2 mM and cells were incubated for 12 h. Plates were washed twice with PBS and reincubated with antibiotic-free medium. After 2.5 h nocodazole (Sigma Co., St Louis, MO) was added to 40 ng/ml and 1 h later, loosely attached cells were removed by aspiration after mechanical shake off. Adhering cells were reincubated in 75 ml medium with 40 ng/ml nocodazole for up to 4 h, mitotic cells were harvested after mechanical shake off. The mitotic index of detached cells was determined by staining nuclei with the dye Hoechst 33258 and was >95%.

Antisera

A polyclonal rabbit antisera against recombinant H-rab4 was raised and blot-purified as described previously (van der Sluijs *et al.*, 1991). An antibody against the synthetic C-terminal peptide K-L-R-Q-L-R-S-P-R-R-T-Q-A-P-N-A-Q was raised in rabbits that were injected with KLH-conjugated peptide. The antiserum was affinity-purified on the C-terminal peptide immobilized on activated Sepharose 4B. A mouse monoclonal antibody against the human transferrin receptor was obtained from ATTC (Bethesda, MD).

Construction of mutant rab4

A cDNA encoding the full length human protein was digested with *Eco*RI restricted as described by van der Sluijs *et al.* (1991) and subcloned into Bluescript KS^+ (Stratagene, La Jolla, CA). The point mutants rab4–S^{196}Q and rab4–S^{196}D, in which Ser196 was changed to Gln and Asp, were created by site directed mutagenesis (Kunkel, 1985) using the mutagenic oligonucleotides 5′-TTG-AGA-CAG-CTG-AGG-CAA-CCG-CGG-CGC-ACC-CAG-3′ and 5′-TTG-AGA-CAG-CTG-AGG-GAC-CCG-CGG-CGC-ACC-CAG-3′ respectively (base changes are underlined). To create the truncation mutant rab4–CT3, in which the three C-terminal amino acids were deleted, wild type rab4 cDNA in Bluescript KS^+ was linearized with *Kpn*I. A stop codon in position 207 was generated with the polymerase chain reaction using the mutagenic primer 5′-TCT-AGG-AAT-TCC-TCT-CCT-AAC-AAC-CTC-ACT-CCT-GAG-CG-3′ and the extended Bluescript KS primer 5′-CTC-GAG-GTC-GAC-GGT-ATC-GAT-AAG-3′. The PCR product was gel purified, digested with *Eco*RI and cloned into the *Eco*RI site of the mammalian expression vector pFRSV. All mutations were confirmed by dideoxy sequencing of the entire cDNA (Sanger *et al.*, 1977).

Scanning laser confocal immune fluorescence microscopy

Cells were seeded on 12 mm coverslips and grown to 50% confluency for 2 days. The cells were permeabilized for 5 min at room temperature with 0.5% saponin, 5 mM EGTA, 1 mM $MgSO_4$, 80 mM potassium PIPES pH 6.8 (Chavrier *et al.*, 1990), fixed with 2% paraformaldehyde and blocked with goat serum as described (van der Sluijs *et al.*, 1991). The cells were double labelled with an affinity-purified rabbit anti-rab4 antibody (1:100) and the mouse monoclonal antibody OKT9 against the human transferrin receptor (1:100). The cells were washed three times for 10 min with 0.1% saponin in PBS containing 10% goat serum and stained with 1:50 dilutions of TRITC labelled donkey anti-rabbit and FITC labelled goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, Westgrove, PA). Coverslips were mounted in Moviol (Calbiochem, San Diego, CA) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (Sigma Co., St Louis, MO) to reduce photobleaching by the 15 mW Krypton laser. The coverslips were viewed on a Bio-Rad MRC-600 confocal imaging system mounted on a Zeiss Axiovert microscope using a 63 × Zeiss objective. Images from both channels were stored on a magneto optical disk and merged with the Bio-Rad software. Pictures were taken directly from the monitor screen on Ektachrome HC100 film.

Stable expression in CHO cells

cDNAs encoding wild type rab4, rab4–S^{196}Q, rab4–S^{196}D or rab4–CT3 were subcloned in the *Eco*RI site of the amplifiable expression vector pFRSV (Miettinen *et al.*, 1989) and transfected using the calcium phosphate method. Stably transfected cells were selected, cloned and amplified in methotrexate-

containing media (final concentration 60 μM). Stable double transfectants, also expressing the human transferrin receptor were generated as described (van der Sluijs *et al.*, 1992) and maintained in the same medium except that G418 was added to 0.6 mg/ml. Expression of protein was analysed by immunofluorescence, Western blot and immune precipitation.

In vivo phosphorylation

Mitotic cells were washed twice in phosphate-free DMEM (Sigma Co., St Louis, MO), containing 40 ng/ml nocodazole and starved for 30 min at 37°C. Cells were subsequently centrifuged, resuspended to $5 \times 10^6 - 1 \times 10^7$/ml in P_i-free DMEM and labelled for 30 min with 0.5–1 mCi/ml orthophosphate $^{32}P_i$. Reversibility of phosphorylation was investigated by prelabelling mitotic cells as previously described. Cells were then washed twice with ice-cold nocodazole-free P_i-free DMEM and incubated for another hour at 37°C in the same medium containing 1 mCi/ml $^{32}P_i$. Cells were washed in ice-cold wash buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 25 mM sodium β-glycerophosphate and 50 μM sodium vanadate) lysed in wash buffer containing 0.5% Triton X-100 and 1 mM PMSF, and processed for immune precipitation. Alternatively, cell pellets received 5 vol of wash buffer and were processed for subcellular fractionation and subsequent immune precipitation. Non-synchronized cultures or cells adhering to the tissue culture plate after the mitotic shake-off were used as interphase controls.

In vitro phosphorylation

Sf9 cells infected with recombinant baculovirus encoding human $p34^{cdc2}$ kinase only or both human $p34^{cdc2}$ kinase and cyclin B, were generated as described (Parker *et al.*, 1991). Cells (3×10^6) were pelleted and frozen at −80°C. The extracts were prepared by adding 20 μl 20 mM Tris–HCl pH 7.4, 1 mM DTT to defrosted cell pellets, which were then shaken for 10 min at 4°C. The extract was then spun for 15 min at 13 000 *g* in a cooled microcentrifuge. The supernatant was either used immediately for *in vitro* phosphorylation or stored in liquid nitrogen. In some experiments, Sf9 supernatant was pretreated with $p13^{suc1}$ beads (Dunphy *et al.*, 1988). *In vitro* phosphorylation was done for 25 min at 30°C in a final volume of 30 μl containing 10 mM $MgCl_2$, 50 mM Tris–HCl pH 7.4, 80 mM sodium β-glycerophosphate, 6 mM EGTA, 1 μg recombinant human rab4, 10 μM ATP, 10 μCi [γ-^{32}P]ATP and 1 μl Sf9 cell supernatant. Kinase reactions were stopped by adding wash buffer containing 0.5% Triton X-100 (see *In vivo* phosphorylation). rab4 phosphorylation was assayed by immune precipitation with an anti-rab4 antibody and SDS–PAGE on 12.5% minigels.

Isoprenylation in mitotic cells

Lovastatin (MSD, Caguas, PR) was added to 50 μM 2 h before the shake off procedure. During the last hour the concentration was increased to 100 μM. After shake off, detached cells were washed twice with αMEM containing 5% dialysed FCS, 40 ng/ml nocodazole and 100 μM lovastatin (isoprenylation medium). Cells were labelled for 2 h at 37°C in isoprenylation medium containing 150 μCi/ml RS-[5-^{3}H(N)]mevalonolactone (NEN, Boston, MS). Interphase cells were labelled overnight in the same medium containing 50 μM lovastatin, without nocodazole. Cells were washed twice with PBS and once in TEAS250, and processed for subcellular fractionation and immune precipitation experiments.

Carboxymethylation in mitotic cells

Mitotic cells were washed twice with methionine-free DMEM containing 40 ng/ml nocodazole and starved in this medium during a 30 min incubation. Subsequently cells were resuspended to 10^7 cells/ml and labelled for 2 h in methionine-free DMEM containing 750 μCi/ml of L-[methyl-^{3}H]-methionine (NEN, Boston, MA). rab4 was immunoprecipitated and carboxymethylation was quantified by base hydrolysis as described (Chelsky *et al.*, 1987). Interphase cells were labelled in the same medium without nocodazole.

Subcellular fractionation

Cells were broken by rapid freeze–thaw, broken cells were resuspended in buffer and centrifuged for 15 min at 3500 r.p.m. in a cooled microcentrifuge to generate a post-nuclear supernatant. A high speed supernatant and membrane pellet were obtained after centrifugation of the post-nuclear supernatant for 45 min at 145 000 *g* in a TLS55 rotor. Membrane and cytoplasmic fractions were either analysed by immune precipitation or Western blotting.

Immune precipitation and SDS–PAGE

For immune precipitations, rabbit antiserum was incubated 1 h with protein A–Sepharose CL4B beads at 4°C. Antibody-coated beads were washed with 0.5% Triton X-100 in PBS and resuspended in lysis buffer containing 0.5% Triton X-100. The complexes were then incubated with lysate for 1 h at 4°C and washed four times with RIPA buffer (100 mM Tris–HCl pH 8.3, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 25 mM sodium β-glycerophosphate, 50 μM Na vanadate and 1 mM PMSF). Washed pellets were resuspended in 20 μl 10 mM Tris–HCl and 5 mM EDTA pH 6.8, to which an equal volume of 2 × Laemmli sample buffer was added. Samples were boiled 5 min at 95°C, shaken for 5 min and centrifuged for 3 min at 13 000 r.p.m. in a microfuge. Supernatants were electrophoresed on 12.5% minigels (Bio-Rad, Richmond, CA) and subjected to fluorography or autoradiography. Exposure times were between 2 days and 5 weeks for ^{3}H samples and 2–24 h for ^{32}P samples.

2D gel electrophoresis, [α-^{32}P]GTP overlay and Western blotting

Samples were resolved by a combination of high resolution IEF and SDS–PAGE essentially as described by Celis *et al.* (1990) and Wandinger-Ness *et al.* (1990). Gels were washed twice for 15 min with 50 mM Tris-HCl pH 7.5 and 20% glycerol, following which the proteins were transferred to nitrocellulose in 10 mM $NaHCO_3$, 3 mM Na_2CO_3 pH 9.8 using a modification (L.Huber and K.Simons, in preparation) of a previously described method (Lapetina and Reep, 1987). Nitrocellulose filters were rinsed for 30 min in NaH_2PO_4 pH 7.5, 10 μM $MgCl_2$, 2 mM DTT and 4 μM ATP (binding buffer). Transfers were then incubated for 120 min with 1 μCi/ml [α-^{32}P]GTP in binding buffer, extensively washed with the same buffer and air dried. [α-^{32}P]GTP binding was visualized by autoradiography using an exposure time of 24 h. After elution of [α-^{32}P]GTP from the nitrocellulose (L.Huber and K.Simons, in preparation) we used the same transfers for Western blotting with the anti-peptide antibody. Detection on the transfers of 2D gels was done with HRP-conjugated goat anti-rabbit antibody followed by chemiluminescence. Western blotting of nitrocellulose transfers from regular SDS–PAGE gels was performed exactly as described by van der Sluijs *et al.* (1991).

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